LESSON 23:
PRODUCTION OF SECONDARY METABOLITES

Introduction

Productivity

Several products were found to be accumulated in cultured cells at a higher level than those in native plants through optimization of cultural conditions (Table 2 of previous Lesson). For example, ginsenosides by Panax ginseng, rosmarinic acid by Colleus blumei, shikonin by Lithospermum erythrorhizon , diosgenin by Dioscorea, ubiquinone-10 by Nicotiana tabacum were accumulated in much higher levels in cultured cells than in the intact plants.

However, many reports have described that yields of desired products were very low or sometimes not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells. In order to obtain products in concentrations high enough for commercial manufacturing, therefore, many efforts have been made to stimulate or restore biosynthetic activities of cultured cells using various methods. The following are typical approaches that may increase productivity of cultured plant cells.

Optimization of Cultural Conditions

Medium

A number of chemical and physical factors affecting cultivation have been tested extensively with various plant cells. These factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. This is the most fundamental approach in plant cell culture technology. Since there are many reports and patents concerning optimization of cultural conditions in order to improve growth rates of cells and/or higher yield of desirable products, it is impossible to give detailed results in this section. Therefore, only a few typical examples will be described.

Zenk et al. tested various well-known basal media for the production of serpentine, an indole alkaloid, as summarized in Table 1. The results indicate that the amount of serpentine depends on the composition of the basal medium used. Among them, Murashige-Skoog's (MS) formulation was recognized to be the most suitable one for the production of this particular alkaloid by Catharanthus roseus suspension. This is of course not always true in other cultures.

Table 1. Effects of Different Media on Growth and Serpentine Production in Cell Suspension Cultures of Catharanthus roseus

Basal Medium *	Cell yield g dwt/1	Serpentine mg/1	Serpentine Content % dwt
Blaydes	7.6	4.4	0.06
Gamborg -	4.6	0.5	0.01
B5; + 2,4-	5.2	0	0
D: 1 mg/1	7.6	1.2	0.02
Gamborg	5.1	0	0
+ 2,4 D: 2	5.4	6.6	0.12
mg/1	9.3	0	0
Gamborg	8.9	10.4	0.12
+ NAA :	2.3	2.0	0.09
1.86 mg/1	5.0	0	0
Gamborg	0.8	0	0
Heller +			
IAA:O.175;			
BA: 1.13			
mg/1			
Linsmaier			
and Skoog			
Murashige			
and Skoog			
Nitsch and			
Nitsch			
Velicky and			
Martin			
White			

* IAA = Indole-3-acetic acid
NAA = 1-Naphthalene acetic acid
2,4-D = 2,4-Dichlorophenoxy acetic acid
Kin = Kinetin
BA = Benzyladenine

Source: Zenk, M.H., et al., Plant Tissue Culture and Its Biotechnological Application p. 27 (1977), Springer-Verland, Berlin, Heidelberg.

Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of Salvia officinalis was 3.5 g/L when 5% of sucrose was used but it was 0.7 g/L in the medium containing 3% sucrose.

Among a number of other components in the medium phytohormones such as auxins and kinetins have shown the most remarkable effects on growth and productivity of plant metabolites. In general, an increase of auxin levels, such as 2,4-D, in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites. This is why auxins are commonly added to the medium for

callus induction, but they are added at a low concentration or omitted for production of metabolites. Decendit reported that cytokinins stimulated alkaloid synthesis which was induced by removing auxin from the medium of a cell line of C. roseus. However, productions of L-DOPA by Mucuna pruriens, ubiquinone-10 by N. tabacum and diosgenin by Dioscorea deltoidea were stimulated by high levels of 2,4-D.

Although kinetin is one of the most popular cytokinins, 4-chloro-2-diphenylurea was reported to stimulate production of an antitumor compound, tripdiolide, by Triptorygium wilfordii cell cultures. Gibberellic acid is also effective on plant cell cultures. DiCosmo et al. recently reported that the growth of callus of a taxol-producing plant, Taxus cuspidata, was significantly promoted by addition of gibberellic acid into the solid medium.

Temperature, pH, Light and Oxygen

The effects of temperature, pH, light and oxygen are all parameters that must be examined in the studies of secondary metabolites production. A temperature of 17- 25° C is normally used for induction of callus tissues and growth of cultured cells. But, each plant species may favor a different temperature. Toivonen found that lowering the cultivation temperature increased the total fatty acid content per cell in dry weight.

The medium pH is usually adjusted to between 5 and 6 before autoclaving and extremes of pH are avoided. The optimum pH is determined and controlled using a small scale bioreactor or a jar fermentor with pH control equipment.

Present scale-up technology dictates the use of stainless steel tanks for growth of plant cells on an industrial scale, thus in general, eliminating the use of light. However, since there are cases of light-stimulated secondary metabolites production, this factor should be investigated as it could help elucidate regulatory factors. Modification of fermentors with lighting facilities have also been carried out.

As described in the section on Facilities, various fermentors have been designed and tested by many researchers. These include modification of impellers or agitators for microbial fermentors, improvement of air-lift fermentors and use of new type reactors such as rotary-drum type fermentors. For hairy root cultures, fermentors equipped with special hangers inside the vessel are being used.

Each plant species has different optimized conditions both for growth of the cells and for production of useful products, so it is necessary to optimize the conditions in each case.

High Cell Density Culture

To increase the productivity of secondary metabolites, high cell density cultures have been investigated. Using a newly designed fermentor and optimized culture medium, Coptis japonica cells were grown up to 75 g/L of cell mass. The highest yield of berberine, 3.5 g/L, was produced intracellularly in 55 g/L of the cell mass.

Absorption of Products

Most products are generally accumulated intracellularly by cultured plant cells, but some compounds were reported to be secreted into the media. Chinchona ledgerina cells excrete anthraquinones in the liquid medium. Robins et al. reported that addition of a resin, XAD-7, into its suspension culture stimulated the production of anthraquinones up to 539 mg/L which was approximately a 15 times increase compared to the medium without resin. The pigments were mostly found to be absorbed by the resin. The yields of ajmalicine and serpentine produced by C. roseus were also increased by addition of XAD-7 and the ratio between both alkaloids produced was changed. It is of interest that production of these alkaloids which are known to accumulate inside cells were affected by the presence of resin. A similar approach was conducted by Knoop et al. in which addition of active charcoal in the medium stimulated the yield of coniferyl alcohol up to 60-fold in a Maticaria chamomila culture.

Becker et al. recognized increases of several plant products produced using a continuous extraction process with two-phase organic solvents.

Selection of High-Producing Strains

The physiological characteristics of individual plant cells are not always uniform. For example, pigment producing cell aggregates typically consist of producing cells and non-producing cells. In 1976, Zenk and his colleagues in Germany obtained cell lines of Catharanthus roseus which accumulated higher levels of ajmalicine and serpentine as determined by radioimmunoassay. This is similar to monocolony isolation of bacteria. Following their excellent results, a number of researchers have used cell cloning methods as this is the most promising way of increasing the levels metabolites present. Some typical examples are shown in Table 2. Most of them are related to production of pigments, such as anthocyanins, as visual selection is easy because of the color.

Table 2. Typical examples of Cell Cloning Application

Products	Plants	Factors
Anthocyanins	Vitis hybrid	2.3-4
Anthocyanins	Euphorbia milli	7
Berberine	Coptis japonica	2
Biotin	Lavendula vera	9
Ubiquinone-10	Nicotiana tabacum	15

Source: Misawa, M. Advances in Biochemical Engineering/ Biotechnology, Vol.31, Ed. Fiechter, A. p. 70 (1985), Springer-Verlag, Berlin, Heidelberg.

Yamakawa et al.isolated a strain of Vitis hybrid using an agar plating feeder method; the culture produced 3.4% (dry wt.) of anthocyanins. A strain of Euphorbia milli was also recognized to accumulate about 7 times higher amounts of anthocyanins than that of the parent strain after 24 selections. Statistical and cell-pedigree analysis proved that production of the red pigments was stable. According to Constabel's investigation with C. roseus, anther, leaf, and meristem explants, synthesis and accumulation of alkaloids in cell cultures differ significantly

provided the comparison is based on individual alkaloid types. Variation of alkaloid profiles in callus tissue developed from the anther walls and fillaments of C. roseus ranged from cell lines with no detectable alkaloids to those with 12 alkaloids representing Corynanthe-, Strychnos-, Aspidosperma-, and Iboga-type alkaloids.

Yamada et al. repeated cell cloning using cell aggregates of Coptis japonica, and obtained a strain which grew faster and produced a higher amount of berberine and cultivated the strain in a 14 L bioreactor. The selected cell line increased growth about 6-fold in 3 weeks and the highest amount of the alkaloid produced was 1.2 g/L of the medium. The strain was very stable, producing a high level of berberine even after 27 generations.

Cultured, green Lavendula vera cells grown in the light were found to accumulate a high level of free biotin by Watanabe et al. To select a high-producing cell line, pimelic acid, a precursor of biotin, was used as a selecting agent. The level of biotin accumulated by a selected cell-line was 0.9 µg/L which was 10 times the amount found in the leaves. Selection of a high ubiquinone-10 producing Nicotiana tabacum strain has produced excellent results. A group in the Japan Tobacco Inc. isolated a number of strains producing high levels of ubiquinone-10 from tobacco cell cultures and analyzed the contents in the cells by HPLC. Several strains producing large amounts of ubiquinone-10 in suspension culture were selected and were subjected to further cell cloning. After the 13th recloning, a strain was selected from approximately 4000 cell clones tested giving a ubiquinone-10 level of 5.2 mg per dry weight of cells. When N. tabacum BY-2, a parent strain used for the cloning, was isolated as a producer in 1976, the titer for ubiquinone-10 was only 360 µg/g dry weight, therefore the level was increased by more than 14 times by selection. It may be noted that 5.2 mg/g corresponds to 180 times the amount produced by the parent plant.

Zieg et al. reported the selection of high pyrethrin producing tissue cultures derived from Chrysanthemum cinerariaefolium and indicated that analytical screening of the tissue lines enabled the selection of a few "high yielding" strains which were derived from high yielding plant selections. A rapid assay method is crucial in the selection of a high yielding cell line. Deus and Zenk reported that a procedure using fluorescence assay to select ajmalicine and other major heteroyohimbine alkaloids producing cells of C. roseus was of clear advantage even over a radioimmunoassay procedure since almost unlimited numbers of colonies could be screened in this way. However, the cell cloning is especially compatible with selection for high pigment production since selection can be achieved visually, or with the use of simple spectrophotometric analysis.

Cell cloning is undoubtedly a very useful technique to increase the level of secondary metabolites and it should be applied as widely as possible. However, it is not obvious why cultures contain both high- and low-yielding cells. Only a few papers concerned with possible mechanisms have appeared. Bohm indicates that the lack of specific enzyme(s) represents the most important reaction for the inability of plant cell cultures to produce secondary metabolites. Berlin et al. compared the highest cinnamoyl putrescine producing, p-flurophenylalanine resistant strain TX-4 or N. tabacum L. CV Xanthi with a low producing strain for five enzymes of the biosynthetic pathway. As a result, activities of these enzymes, phenylalanine ammonia-lyase, trans-cinnamate-4-hydroxylase, 4-coumarate-CoA ligase, ornithine decarboxylase and arginine decarboxylase were found to be 3 to 10 times higher in TX4 cells.

Protoplasts were also used for the selection of high-shikonin producing cell lines of L. erythroryzon and thiophene producing Tagetes patula cell lines.

Addition of Precursors and Biotransformation

Addition of Precursors

Addition to the culture media of appropriate precursors or related compounds sometimes stimulates secondary metabolite production. This approach is advantageous if the precursors are inexpensive. Since Chan and Staba initially examined the production of alkaloids with this approach in the 1960's, many similar experiments have been carried out. For example, amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids, and ephedorin and some stimulative effects have been observed. It is true that some amino acids are precursors of various alkaloids, but generally the biosynthetic steps from amino acids to alkaloids are so complicated that the author doubts whether amino acids added were incorporated into the alkaloids directly in cell culture. Perhaps, they affected not only alkaloid biosynthesis directly as precursors, but also indirectly through other metabolic pathways in the cells.

Phenylalanine is one of the biosynthetic precursors of rosmarinic acid. Addition of this amino acid to Salvia officialis suspension cultures stimulated the production of rosmarinic acid and shortened the production time as well. Tabata et al. reported that addition of 500 mM tropic acid to the medium of Scopolia japonica increased the amount of alkaloids by up to 14 times. The level of an anticancer compound, tripdiolide, produced by T. wilfordii cultured cells was increased by addition of 100 µg/L farnesol which is dephosphorylated farnesyl pyrophosphate, and an intermediate in the biosynthesis of terpenoids. Addition of phenylalanine into the agar medium of Taxus cupsidata cells was found by DiCosmo et al. to stimulate the biosynthesis of an anticancer compound, taxol.

However, biotransformation using intact cells or immobilized cells is an alternative way of producing a product by adding precursors into the culture media.

Endress et al. reported that Rauwolfia serpentina formed a new indole alkaloid, 6-hydroxytaumacline, in significant amounts when the cells were cultivated in the presence of ajmalicine. Arbutin, a skin depigmentation agent, is produced by biotransformation of hydroquinone using C. roseus cells. Addition of the precursor into the liquid culture medium of this cell line produced arbutin efficiently. As described later, this process is being studied by Shiseido in Japan for commercial application.

In order to produce a prodrug, Umetani et al. studied the glucosylation of umebelliferone and salicylic acid using Mallotus japonicus cells and reported that the latter was converted to its

o-glucoside with a yield of 90-95%. The maximum level of the product obtained was 0.9 g/L. The glucoside showed as potent an analgesic activity as salicylic acid, while its effect was more rapid and more long-lived than that of salicylic acid in mice. It is of interest that plant cells are able to form prodrugs having commercial usefulness. Dombrowski & Alfermann also worked on glucosylation of salicylic acid and its derivatives. They found that cultured Salix matsudana cells glucosylated salicylic acid to 2-o-salicylic acid-ß-D-glucoside. The cells also converted salicyl alcohol to both salicin (2-o-salicyl alcohol-ß-D-glucoside) and isosalicylicin (1-o-salicyl alcohol-ß-D-glucoside). However, salicylic aldehyde was converted to ß-D-glucosides of salicylic acid and salicyl alcohol instead of helicin.

The conversion of geraniol and nerol to neral and geranial by Vitis vinifera cell suspension cultures is also interesting. Using plant cell culture techniques, radioactive labelled compounds can be formed from appropriate substrates as reported by Mangold et al. This is a useful application of the technology because of high value of the product.

Biotransformation

Instead of the addition of a particular compound as a precursor into the culture medium of plant cells, a suitable substrate compound may be biotransformed to a desired product using plant cells. This approach has been extensively applied in the fermentation industry using microorganisms and their enzymes. For example, L-aspartic acid and L-malic acid are being manufactured commercially from fumaric acid, respectively using microorganisms. And various steroids are also produced by microbial biotransformations.

Biotransformation of ß-methyldigitoxin to ß-methyldigoxin using D. lanata cells has been extensively investigated by Reinhard and Alfemann in Germany since 1974 because digoxin has a large market as a cardiac glycoside. About 600-700 mg of ß-methyldigoxin per litre was obtained using a 200 L reactor. This process was studied for commercialization by Boehringer Mannheim Co.

Allelix Inc. in Canada established processes for production of a very expensive antitumor drug, vinblastine, from catharanthine and vindoline using a biotransformation as well as a simple chemical synthesis which will be described later. The process is now being developed for commercialization by Mitsui Petrochemical in Japan. Concerning vinblastine-related compounds production, Bede and DiCosmo also reported a biotransformation of catharanthine and vindoline to anhydrovinblastine using horseradish peroxidase and glucose oxidase mediated coupling of vindoline and catharanthine.

Biotransformation processes including addition of substrates into the cultures are one of the most commercially realistic approaches in plant tissue cultures because of economic reasons. However, the availability of inexpensive precursors is a key issue.

Elicitor Treatment

Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. The best understood systems are those of fungal pathogens in which case the regulatory molecules have been identified as glucan polymers, glycoproteins and low molecular weight organic acids. DiCosmo and Towers reviewed possible correlations between stress and secondary metabolism in cultured cells.

Examples of microbial elicitor induction include psoralen production in parsley diosgenin production in the Mexican yam and many others. Tyler et al.described that a cell line of Papaver somniferm that synthesized and accumulated sanguinarine, a quaternary benzophenanthridine alkaloid when exposed to a homogenate of the fungus Botrytis. A portion of the sanguinarine was released into the culture medium. Sanguinarine extracted from the intact plants is being used for oral hygienic products.

Effects of elicitors on secondary metabolism have been investigated at the enzymatic levels to determine their mode of action. Eilert and Wolters added autoclaved culture homogenate of yeast, Rhodotorula rubra into the suspension culture of Ruta graveolens and found that S-adenosyl-L-methionine: anthranilic acid N-methyltransferase was elicited. A yeast polysaccharide preparation induced L-tyrosine decarboxylase in suspension cultures of Thalictrum rugosum and Eschscholtzia californica; the enzyme was induced after 5 hours after addition of the elicitor at 30 to 40 µg/g-cell fresh wt.

Recently Mizukami et al. reported a transient increase in rosmarinic acid, a-0-caffeoyl-3, 4-dihydroxyphenyllactic acid, content in cultured cells of L.erythrorhizon after addition of yeast extract to the suspension cultures: a maximum was reached in 24 hr. When the plant cells were treated with yeast extract on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times and the activity of phenylalanine ammonia-lyase in the cells rapidly increased before synthesis of rosmarinic acid.

In suspension cultured cells of parsley, Petroselinum crispum, Conrath et al. found that chitosan elicited a rapid deposition of the 1,3-ß-glucan, callose.

Recent developments in phytochemical elicitation have shown that simple inorganic and organic molecules can induce product accumulation. Sodium orthovanadate and vanadyl sulphate induced the accumulation of isoflavone glucosides in Vigna angularis cultures and indole alkaloid accumulation in Catharanthus roseus cultures, respectively. Other substances found to stimulate alkaloid accumulation in C. roseus include sodium chloride, potassium chloride and sorbitol as well as abscisic acid. Processes such as these, employing simple and cheap elicitors have much promise in industrial scale plant cell cultures.

Davis et al. recognized that addition of oxalate to the medium of Gossypium hirsutum suspension culture could reduce the amount of Verticillium dahliae elicitor to be employed to stimulate metabolite synthesis. Addition of a fungal elicitor often inhibits the growth of plant cells but a combination of the elicitor and oxalate did not reduce the cell mass of the plant, therefore secondary metabolite synthesis was increased up to ten fold.

Dunlop and Curtis reported that a combination of phosphate limitation and fungal elicitation synergistically increased production of secondary metabolites. They found that either

phosphate limitation or elicitation with a mycelial extract of the fungus, Rhizoctonia solani alone results in increased production of the sesquiterpene solavetivone by Agrobacterium rhizogenes-transformed hairy root cultures of Hyoscyamus muticus. However, when phosphate limitation is coupled with fungal elicitation, the productivity increase is considerably greater than that obtained with either method alone.

Although the mechanism by which elicitors increase the productivity of secondary plant metabolites has not been elucidated, their stimulating activity is quite significant if an appropriate elicitor is chosen to stimulate synthesis of a particular product. However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated in a fermentor separately from cultivation of plant cells using another fermentor. The fermentation cost for an elicitor-producing microorganism is not always inexpensive. In this sense, a simple and cheap compound should be employed as an elicitor.

Application of Immobilized Cells

Immobilization of plant cells is considered to be of importance in research and development in plant cell cultures, because of the potential benefits that could be provided:

a. The extended viability of cells in the stationary (and producing) stage, enabling maintenance of biomass over a prolonged time period
b. Simplified downstream processing (if products are secreted)
c. The (putative) promotion of differentiation, linked with enhanced secondary metabolism
d. Higher cell density enabling a reduced bioreactor size, thereby reducing costs and the risk of contamination
e. Reduced shear sensitivity (especially with entrapped cells)
f. Promotion of secondary metabolite secretion, in some cases
g. Flow-through reactors can be used enabling greater flow rates
h. Minimization of fluid viscosity increase, which in cell suspension causes mixing and aeration problems.

An immobilization system which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form, could dramatically reduce the costs of phytochemicals production in plant cell culture. However, an immobilized system also has the problems described below:

a. Immobilization is normally limited to cases where production is decoupled from cell growth
b. The initial biomass must be grown in suspension
c. Secretion of product into the extracellularly medium is imperative
d. Where secretion occurs there may be problems of extracellular degradation of the products
e. When gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

Due to these problems, a system with commercial potential has not yet been developed in plant tissue cultures. However, various immobilization methods have been developed, ie. entrapment, adsorption and covalent coupling.

Some preliminary results have been obtained with immobilized cells. Early work with C. roseus, showed that agar, agarose and carageenan were all suitable immobilization matrices suitable for maintenance of cell viability; but alginate was superior in terms of ajmalicine production. The accumulation of serpentine by C. roseus and anthraquinones by Morinda citrifolia were both enhanced in the immobilized state when compared with freely suspended cells. It should be noted however, that the possibility that alginate acts as an elicitor of secondary metabolism cannot be ruled out. Agar has been shown to stimulate shikonin accumulation in L. erythrorhizon cultures. Lambe and Rosevear have successfully immobilized C. roseus cells in polyacrylamide with alginate and observed prolonged viability and increased productivity.

Adsorption immobilization has been successfully used with a number of plant species. Capsicum frutescens cells immobilized on polyurethane foam produced 50 times as much capsaicin as suspension cells. Similarly, Solanum nigrum cells accumulated glycoalkaloids to levels exceeding those found in suspensions. Datura innoxia cells accumulated tropane alkaloids with a profile similar to that of the intact plant, whilst in free suspensions productivity was markedly suppressed. In general, it appears that mild immobilization either through gel entrapment or surface adsorption enhances productivity and prolongs the viability of cultured cells.

As described in the section on Biotransformation, immobilized cells can also be used as biocatalysts for biotransformations. Such a system compares favourably with the use of freely suspended cells since, in the case of immobilization, the catalyst is theoretically reusable and the product is easily separated from the biomass. The most appropriate example is that of the 12-hydroxylation of ß-methyldigitoxin to ß-methyldigoxin with alginate-entrapped Digitalis lanata cells. The enzyme activity was maintained by the immobilized cultures for a period of 61 days. Furthermore, the product was located in the extracellular medium. Mild permeabilization of the cells may enable biotransformation rates to be increased. Polyurethane-immobilized C. frutescens cells fed capsaicin precursors produced this metabolite at levels of up to 10 times those of non-fed cultures. DiCosmo et al. found that glass fibres can be used as a carrier of plant cells to produce useful plant metabolites. Papaver somniforum cells were immobilized on fabric of loosely woven polyester fibres arranged in a spiral configuration on stainless steel support frame by Kurz et al. to produce sanguinarine, an antibiotic in oral hygiene. The yield was 3.6 mg/g-fw by immobilized cells and was more than twice as much as by suspension cells.

Product Secretion

Many plant products produced by cell cultures have been reported to be accumulated intracellularly. However, it may be possible to produce much higher level of product if it was secreted into the medium. This is because the product intracellularly accumulated sometimes inhibits its own synthesis by regulation mechanisms such as product inhibition and repression. For many immobilized plant cell systems to work it is essential that a significant amount of product is released into the medium. Two types of immobilization system with C.

roseus i.e. gel entrapment in polysaccharide beads and in polyacrylamide sheets, have both exhibited alkaloid release by mechanisms that do not appear to be associated with losses in viability. Capsicum frutescens cells immobilized on polyurethane released capsaicin entirely into the medium, although other species immobilized by the same method retained the product intracellularly.

To enhance release, permeabilization of cell membranes has been attempted, but with only limited success. Brodelius tested five permeabilizing agents on three different species, and although product release was achieved, cell viability dropped in most cases. The exceptions were DMSO and Triton X-100, applied to C. roseus cells. Other attempts to permealize cells have also resulted in non-viable populations and the use of electroporation has also resulted in a viability decrease. The release of betanin by Beta vulgaris cells ultrasonicated for 20 to 60 seconds has been reported, with no apparent effect on cell viability. Chenopodium rubrum cells, immobilized in alginate beads, secreted the red betacyanin pigment amaranthin into the medium. However, the pigment was subsequently degraded; chitosan and DMSO permitted further product release into the extracellular medium, but this was also accompanied by product degradation. Low concentrations of chitosan (0.01%) and DMSO (5.7%) incubated with the cultures for 96 hr did not appear to affect viability significantly, but a longer incubation period (196 hr) had a deterlerious effect.

The lack of an appropriate means of product release is a serious problem in terms of an industrial approach particularly based on immobilization of plant cells. It is perhaps necessary to examine in vivo physiological mechanisms of release. Thus, some researchers have been investigating the factors involved in vacuolar phytochemical storage. The accumulation of indole alkaloids in C. roseus vacuoles has been attributed to an ion-trap mechanism whereby the basic indole alkaloids are trapped in the acidic vacuole due to their positive charge at low pH, preventing diffusion across the tonoplast. This mechanism has also been demonstrated for quinoline alkaloid accumulation in Cinchona species.

Active uptake mechanisms have also been reported for indole alkaloids in C. roseus vacuoles and isoquinoline alkaloids in Fumaria capreolata L. The physiological significance of these two possible mechanisms is not yet clear, since there is obviously strong evidence for both. In terms of product release, it is pertinent to note that in cell cultures, an efflux of alkaloids was observed under certain conditions, indicating an equilibrium between the intracellular and extracellular compartments that could be perturbed by medium acidification with subsequent product release. The release of serpentine by C. roseus cells was observed when the cells were filtered and resuspended in fresh or conditioned medium and it was suggested that temporary membrane uncoupling was responsible.

Although most plant cell culture products were shown to be accumulated intracellularly, Shuler et al. recently reported that almost all of taxol produced by Taxus brevifolia cell cultures was detected in the culture filtrate. This is an exceptional case in plant tissue culture research.

Mutagenesis

In the fermentation industry, induction of genetic mutant strains of microorganisms is ubiquitous, and auxotrophic and/or regulatory mutants are used extensively to produce a variety of products including amino acids, nucleotides, antibiotics, etc. However, mutagenesis has limited applicability to plant cell cultures, because of their diploid genetic make-up: the chance of obtaining a double mutation in a target gene is less than 1 in 10^6. Although, in principle, haploid plants can be produced from anther cultures; in practice haploid cell cultures tend to revert to the diploid state. This makes the chance of isolating over-producing cells from mutagen treatment of haploid cells very low. Furthermore, biosynthetic pathways of many secondary metabolites and their regulation mechanisms in higher plants are not always understood precisely, therefore, it is also difficult to know what kind of mutants should be induced in order to increase product synthesis.

However, Berlin induced p-fluorophenylalanine resistant cell lines of tobacco cell cultures and found that five lines of N. tabacum and five lines of N. glauca out of 31 resistant cell lines accumulated higher levels of phenolics. He and his colleagues also reported that a resistant strain of N. tabacum produced 6 to 10 times higher levels of cinnamoyl putrescine than that of the parent strain. Fig.1. shows the comparison of enzyme activities involved in the biosynthesis of cinnamoyl putrescines in a parent strain, TX1 and a resistant strain, TX4. Enzyme activities in the p-fluorophenylalanine resistant strain were much higher than those of the parent. Berlin et al. also found that a parent strain of C. roseus produced catharanthine only in the production medium but its tryptophan analog resistant mutant accumulated the same alkaloid even in the growth medium. Among several types of analogues tested, 4-fluorotryptophan was found to be the most efficient of them.

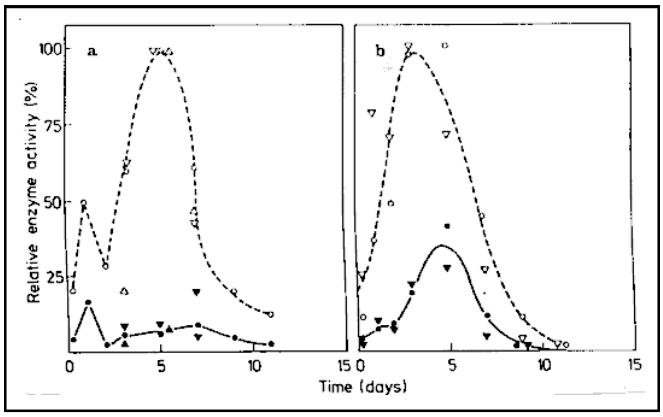

Fig. 1. Comparison of Enzyme Activities involved in the Biosynthesis of Cinnamoyl Putrescines in TX1 (filled symbols) and TX4 (open symbols) of N. tabacum

Source: Misawa, M., In "Adv. in Biochem. Eng./Biotech." Ed. Fiechter, A. p. 73 (1985). Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

Increase of metabolite levels using regulatory mutants is theoretically possible and selection of suitable analogues for this purpose could be an important factor in order to produce a variety of products. Several research groups have used mutagens

such as N-methyl-N'-nitro-N-nitrosoguanidine, ethylmethane sulfonate, N-ethyl-N-nitrosourea or X-rays to induce high yielding strains. In China, a fine callus strain of Anisodus acutangulus was derived through irradiation with 4000 R of X-ray, and the level of scopolamine in the cells was 0.177 mg/g d.wt, which was about 30% higher than of the parent. The productivity was stable. Deus treated C. roseus with X-rays and obtained a cell line having a high producing activity of serpentine whose level was 2%.

A cell line of Lavendula vera producing a high level of free biotin was obtained by gamma ray irradiation for 1 hr. with ^{60}Co (dose 10 KR h) by Watanabe et al. The line was found to contain 7 times the amount, 0.425 µg/g-fresh wt., of free biotin compared with the original unselected cells, 0.061 µg/g fresh wt., and 4.5 times that found in the leaves.

In spite of the difficulty of obtaining auxotrophic mutants in plant cells, several groups have been actively working with this approach using haploid cells or protoplasts although their aim is not always for plant metabolite production.

Induction of a mutant having altered permeability could also be important, because plant cells generally accumulate their metabolites intracellularly, which is disadvantageous in commercial production because the amount of compounds produced is usually low. If the cells excreted products in large amounts, the product cost would be reduced. According to Berlin's results, Thuja occidentalis excreted monoterpenoids, but the levels in the medium were only 5% of those in the mother plant. The same group reported that Macleya microcarpa cells excreted nearly all the alkaloids detectable in the culture flask. Yamakawa et al. cultivated Tinospora rumphii cells in 14 ml medium and found 0.57 (5.3% in dry weight cells) of isoquinoline alkaloids in the cells and 0.5 mg in the culture filtrate after 7 days cultivation. By replacement of the medium with fresh medium after 3 days of cultivation, 0.50 mg of the alkaloids in the cells, and 1.02 mg in the filtrate were accumulated. It seems then that the products must be excreted to some extent. Unfortunately, the secretion mechanisms for secondary products in higher plant cells have not yet been elucidated and extensive fundamental studies are required before meaningful manipulation can take place.

Morphological Differentiation and Hairy Root Culture

Organ Culture

It is desirable to use morphologically undifferentiated cells for the production of useful metabolites in ways similar to microorganisms and there are many examples which show high productive ability of such cells compared with intact plants as already described. In fact, a callus (undifferentiated cells) of Phytolacca americana which was induced from a stem tissue more than 10 years ago, still produces a high level of an alkaloid, betanine. However, a putative link between differentiation and secondary metabolite accumulation has been proposed by many researchers in this field.

Wienmann discussed that a close correlation existed between the expression of secondary metabolism and morphological and cytological differentiation, but he concluded that it is not yet clear to what extent secondary metabolism depends on the development of specific structures. It is unknown whether these two processes are genetically and/or physiologically linked.

The development of a certain level of differentiation is considered to be important in the successful production of phytochemicals by cell cultures. There are many examples in the literature demonstrating a relationship between differentiation and secondary metabolic accumulation. Hiraoka and Tabata successively transferred the callus of Datura meteloides from a medium with auxins to one without and then cultivated continuously. As seen in Table 3. shoots, stems and roots were differentiated by turn and tropane alkaloid levels increased.

Table 3. Relationship between Productivity of Alkaloids and Differentiations

Plants	Alkaloid concentration (% dry wt.)
Callus	1×10^{-2}
Shoots-forming callus	1.5×10^{-2}
Growing shoots	2×10^{-2}
Roots-forming shoots	3×10^{-2}
Leaf of young plant	1×10^{-1}
Leaf of matured plant	1×10^{-1}

Source: Hiraoka, N, and Tabata, M. Phytochem. 13 1671 (1974)

In Digitalis purpurea cultures, Hagimori et al. showed stimulation of digitalis cardenolides production by organ redifferentiation in callus tissues. A similar phenomena was also found in rotenone formation using Derris elliptica and morphinane alkaloid production using Papaver somniferum. Furuya et al. investigated the correlation between the stage of morphological differentiation and producing ability of the alkaloids using P. somniferumi, and found a green callus which differentiates epidermis or vascular bundles produced the alkaloids. A limited degree of tissue differentiation occurred and the cell contained codeine as a main alkaloid while the level of morphine increase as differentiation progressed.

Root cultures derived from suspension cultured cells of P. bracteatum were shown to produce thebaine in 0.03% yield by Zito and Staba. They also reported that axenic callus and shoot cultures of Pyrethrum cinerarifolium had an ability to produce pyrethrin. They isolated a few high yielding strains which were derived from high yielding plant selections. One isolate accumulated 11.3 mg total pyrethrins per 100 g dry weight but it subsequently differentiated into a shoot culture following the first analysis. Therefore, Zito and Staba concluded that differentiated culture tended to produce more pyrethrins than did callus cultures. Using an established shoot culture derived from the disc floret of the plant, 341.8 mg of pyrethrins per 100 g wt. were obtained.

Ozeki and Komamine presented interesting results on the relationship between differentiation and anthocyanin production using Daucus carota cells. The cells were fractionated by Ficoll density gradient centrifugation. In the density fraction (>14% of Ficoll) somatic embryos were formed in a medium

containing 10^{-7} M zeatin but anthocyanin was scarcely produced. On the other hand, the cells in the lower density fraction (>12% of Ficoll) synthesized anthocyanin in the same medium but formed few embryos. 40 to 50% of the total cells in the higher cell fraction synthesized anthocyanin at a maximum.

The use of organs as opposed to cells or cell aggregates might necessitate an adaptation of cultured scale-up technologies, but in general these are not considered to be insurmountable. Indeed, root cultures of Panax ginseng have successfully been grown up to a volume of 20 KL and high levels of ginsenosides were obtained. A semi-continuous production system using differentiated cultures has been suggested by Fuller in which a series of vats with a 10% aliquot being transferred to the next whilst still in the dividing stage, with the remainder being left to grow and accumulate product.

Hairy Root Culture

An alternative possibility is to induce biochemical differentiation, but suppress morphological differentiation. This would be difficult with an initially heterogenous population and the cells would have to be first selected for homogeneity in shape and metabolism. In fact, using a range of species, selection of regular green aggregates with a spherical shape and with cells of a regular morphology has been achieved. Such aggregates were found to enable high flow rates and easy medium removal, superior to those obtained with more dispersed cultures.

Two reports are worth noting with respect to differentiated cell growth and phytochemical production. It has been observed that the insertion of a very fine platinum or titanium wire into a disorganized callus of Helianthus tuberousus brings about morphological differentiation, whereas control, untreated tissue remained in an undifferentiated state. Should this phenomenon be reproducible in other species it might well serve as a well-defined means of controlling differentiation and possibly secondary metabolism. It was suggested that a charge transfer mechanism was responsible for these observations.

A second discovery (possibly acting by a similar mechanism) has shown that electric currents (1-2 µA) bring about shoot regeneration in callus culture at a rate five times greater than in controls (130). This has been patented as a process for the stimulation of growth and differentiation of plant tissue and for increasing the accumulation of secondary metabolites.

The use of Agrobacterium rhizogenes has been receiving attention recently in secondary metabolism research. It inserts the Ri plasmid into wounded tissue, causing the growth of very fine adventitious roots, so-called “hairy-roots”. These roots can be cultured in hormone-free medium and there are several examples of enhanced accumulation of secondary products, relative to non-transformed tissue.

Flores in the U.S. is a leading scientist in hairy root culture studies, and reported in 1987 that all hairy root clones of Hyoscyamus plants grew faster than ordinary root cultures and produced the similar level of tropane alkaloids to that accumulated in the intact plants. Hamill et al. have shown that 17 day-old cultured hairy roots of Beta vulgaris had twice as much betacyanin and three times as much betaxanthin as seedling roots. On a mg/g dry wt. basis, the concentrations of these betalains were equal to or greater than those reported for storage roots.

Berlin et al. recently reported that hairy root cultures of Lupinus polyphyllus and L. hartweigii produced higher amounts of isoflavine glucosides than those of hormone-dependent cells. They also found that hairy root cultures of Peganum harmala synthesized higher levels of ß-carboline glucoid, ruine and serotonin. Valeriana officinalis var. sambucifolia hairy roots produced 44.3 mg/g-cells d.w. of valepotriates having spasmolytic and sedative activities. The productivity was 0.9 mg/g/day and the concentration was approximately 4 times higher than that of normal roots. Eilert and his colleague transformed Ruta gaveolens leaflets using A. rhizogenes and obtained the hairy root tissue. The tissue produced psoralen, isopimpirellin (methyoxylated furanocoumarins), xanthotoxin, bergapten, dictamunine, fagarine, kokusaginine, edulinine and hydroxyrutacridone epoxide. The concentration of sucrose in the medium affected the levels of these secondary metabolites.

Rhodes and his colleagues in U.K. has been employing hairy root culture systems for production of various chemicals. The levels of nicotine in Nicotiana rustica hairy roots and of betanine in Beta vulgaris were shown by his group to be at comparable or slightly higher than their intact plants. They also regenerated hairy roots from protoplasts of N. rustica hairy root tissues. Hairy root cultures of Datura stramonium and related Datura species were reported by Rhodes et al. to produce hyoscyamine as the major tropane alkaloid and small amounts of other tropanes including atropine and hyoscine (scopolamine). To cultivate hairy roots in large-scale fermentors, Rhodes et al. designed and patented a fermentor comprising a vessel defining a fermentation chamber and a space-filling wire lattice of inoculation points designed to be located within the chamber. At the start of the fermentation process, the fermentation vessel is inoculated with suitable small lengths of hairy roots which then grow to substantially fill the vessel. It has been found to be highly advantageous if the lengths of roots which form the inoculant are distributed throughout the vessel. To achieve this, a space-filling lattice of inoculation points is provided in the fermentation vessel. The inoculating lengths are suspended in a suitable medium which is then passed into the vessel. The inoculant lengths lodge at the inoculation points and thereafter grow in a conventional fashion.

Recently, Berlin et al. compared hairy root cultures with suspension cultures in production of isoflavonoids by Lupinus species and that of harmane alkaloid and serotonin by Peganum species, and concluded the hairy root cultures are the superior to suspension cultures.

The biotechnological application of hairy root cultures is promising for a number of reasons:

1. Stable, high level production
2. Fast auxin-independent growth and,
3. The suitability for adaptation to fermentor systems. It is, therefore one of the most feasible techniques from an industrial point of view.

Conclusion

Most callus and liquid cell suspension cultures metabolizing secondary compounds generally produce low yields of these compounds. This is attributed to the lack of organ differentiation in cell or callus cultures. There is no sufficient compartmentalization of enzymes required for synthesis and accumulation of secondary metabolites. To increase the yield of secondary compounds by undifferentiated tissue or cell cultures, therefore, the application of some special techniques may be necessary which are discussed in the Lesson. These help in increasing the productivity of secondary metabolites.

Questions

1. How will you optimize culture conditions for secondary metabolite synthesis?
2. Write short notes on:
   a. Elicitors
   b. Biotransformation
   c. Hairy root cultures
   d. Application of immobilized cells.

Note